

## FINAL TECHNICAL REPORT

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GRANT TITLE: Characterization of light and nitrogen regulated gene expression pathways in marine diatoms

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OBJECTIVE: To delineate pathways for the modulation of gene expression by marine diatoms in response to environmental cues. To elucidate the extent of light- and nitrogen-dependent transcriptional and translational control on the expression of nitrate reductase (NR), glutamine synthetase (GS) and the fucoxanthin-chlorophyll *a/c* pigment protein (FCP). The products of these genes determine the nitrogen assimilation and light harvesting capacity of marine diatoms and play a central role in the coupling of carbon and nitrogen metabolism in these cells. To characterize the genomic organization and promoter structure of NR, GS, and FCP for eventual identification of light- and nitrogen-responsive promoter elements.

APPROACH: Isolate and characterize nucleic acid probes for and antibodies against NR, GS, and FCP. Apply these probes in conjunction with activity assays to determine changes in transcript and protein abundance in diatom cultures exposed to different light and nitrogen availabilities to reveal the extent to which transcription, translation or post-translational modifications control the expression of NR, GS, and FCP. Utilize the probes to determine the cellular location of these genes and protein products and to isolate genomic clones for characterization of their promoter domains.

ACCOMPLISHMENTS: Biochemical and immunological characterization of NR in the diatom Skeletonema costatum has been completed. This form of NR exhibits unique properties compared to the higher plant enzyme. Although kinetically similar, the S. costatum NR exhibits a high  $K_m$  for  $\text{NO}_3$ , reflecting the high  $\text{NO}_3$  storage capacity previously reported for this species. A low temperature optimum ( $14^\circ\text{C}$ ), with pronounced inactivation occurring above this optimum, is indicative of a thermal instability in the FAD binding site in this NR. Our previous work suggested that the pronounced diurnal fluctuation of NR activity observed in S. costatum is related to light-dependent post-translational modulation of this enzyme. A phosphorylation-dependent inactivation of NR under conditions limiting photosynthesis has been observed in higher plants

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and this phosphorylation has been suggested to be correlated with increased sensitivity to  $Mg^{2+}$  inhibition. Interestingly, the S. costatum NR is 100 fold less sensitive to  $Mg^{2+}$  than the vascular plant enzyme and this inhibition is not correlated with NR activity level. Although our observations cannot rule out a phosphorylation-dependent activity modulation, they do suggest that diurnal variation in NR activity in S. costatum does not occur through phosphorylation of the same domains as proposed for vascular plant NR. We are examining whether direct light absorption by the FAD moiety of NR is important in activity modulation of this enzyme. In contrast to the effects of  $Mg^{2+}$ , diatom NR is sensitive to  $Cu^{2+}$  and  $Fe^{3+}$  inhibition in vitro and in vivo. We are pursuing the feasibility of using NR activity as a bioassay for metal toxicity in the environment. A polyclonal antisera was raised against S. costatum NR and recognizes NR in all diatoms tested with no observable cross-reactivity with NRs from green algae, cyanobacteria or prymnesiophytes. A disparity was observed in NR immunoreactive polypeptides in the pennate diatom Phaeodactylum tricornutum, indicating structural divergence of NR among the diatoms as well as between major phytoplankton taxa. A manuscript describing this work has been accepted for publication in *PLANT PHYSIOLOGY*.

The structural divergence of NR revealed by the immunological and biochemical data is even more evident in its gene sequence and has hindered our progress on characterization of the gene. Amino acid sequence alignments reveal 50-60% amino acid identity over limited domains of NRs from fungi, green algae and higher plants. PCR amplification of genomic DNA and cDNA from the seagrass Zostera marina and marine unicellular chlorophyte Dunaliella tertiolecta, using oligonucleotide primers based on conserved amino acid sequences encompassing the cytochrome b and FAD binding domains of NR, yield clear products of the predicted size with high nucleic acid and deduced amino acid sequence identity (53% and 43%, respectively) with the corresponding domain of higher plant NRs. In contrast, the same amplification primers yield an NR product from S. costatum that exhibits only ca. 40% nucleic acid and 25% amino acid identity with known NRs. Alignments based on amino acid chemical and structural similarity reveal only a ca. 45% similarity to known NRs indicating the potential for strong codon and amino acid bias in this diatom gene. Northern analysis revealed that the corresponding transcript (ca. 5 kb) in S. costatum is light and nitrate inducible (light>>nitrate) providing functional evidence for the identity of this clone. Diurnal sampling also indicated that in the diatoms and green alga the NR transcript accumulates only in the light. Southern analysis with the green plant and algal NR probes using low stringency hybridization conditions (detection of sequences with <70% base mismatch) yielded no cross hybridization with DNA from three species of diatoms, a coccolithophore, and the kelp Macrocystis pyrifera, although homologous sequences were identified in Arabidopsis thaliana genomic DNA under these conditions. These results demonstrate dramatically the lack of contiguous nucleic acid sequence in diatoms and other chromophytic algae which shares even limited identity with green plant NR. We are awaiting amino

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acid sequence (N-terminal and internal) data from our purified S. costatum NR to independently verify the identity of the NR clones and redesign probes to complete analysis of NR gene structure in diatoms.

Genes encoding FCP in S. costatum have been characterized through PCR amplification of cDNA. To date four discrete coding sequences have been isolated and further verified using 3' amplification of their corresponding mRNAs. These genes exhibit an average of 70% identity with the genes isolated from the diatom Phaeodactylum tricornutum, and range from 70 to 90% identity in coding sequence among members of the gene family in S. costatum. The 3' untranslated sequences of the corresponding transcripts vary from 200 - 400 bp in length and exhibit limited sequence conservation, making them attractive sites for isolation of gene- and taxon-specific probes. FCP coding sequences that have been amplified from the diatom Thalassiosira pseudonana are intermediate in amino acid sequence identity to FCPs in S. costatum and P. tricornutum. Northern analysis reveals a strong diurnal regulation of FCP transcript (1.1 -1.2 kb) abundance, with transcripts accumulating during the light period and declining during the dark. These observations, in conjunction with previous work, indicate that the expression of these genes is light-dependent in coarse control (ON/OFF) with photosynthetic photon flux density modulating (UP/DOWN) expression level during the light period, thereby leading to enhancement of light-harvesting efficiency in low light conditions. Isolation of FCP cDNA sequences from night and day samples of S. costatum also indicates that FCP genes may be expressed differentially during the photoperiod. Since the primers to the coding sequence of FCP appear to function in a range of diatom species, reflecting the high degree of sequence conservation exhibited by this gene family, we plan to develop a PCR assay to quantify diatom abundance in natural samples based on amplification of this gene. A manuscript describing the characterization of the FCP gene family in Skeletonema costatum is in preparation.

GS coding sequences have been obtained by PCR amplification of S. costatum and D. tertiolecta oligo d(T) primed cDNAs. These clones exhibit high amino acid sequence similarity (45-50%) to known GS sequences and contain conserved amino acid domains characteristic of this protein. The S. costatum clone is nuclear encoded and encodes a 1.2 kb transcript in  $\text{NO}_3$  grown cells. Sequence alignments suggest that this clone is more similar to the chloroplastic GS isoform. Experiments are in progress using 3' and 5' amplications of cDNAs synthesized from mRNA isolated from  $\text{NH}_4$  and  $\text{NO}_3$  grown cells in order to determine the number of GS genes present in S. costatum.

Experiments examining the interactions between light and nitrogen source availability on the expression of NR, GS and FCP have been conducted with S. costatum. These studies indicate an up-regulation of a putative chloroplastic form of GS during  $\text{NO}_3^-$  assimilation with a cytosolic GS activity being induced during supply of external  $\text{NH}_4^+$ . NR activity is induced in the presence of  $\text{NO}_3^-$ .

yet the immunologically detectable protein abundance did not increase to the same extent, corroborating our previous observations that NR in S. costatum may be subject to light and or  $\text{NO}_3^-$  - dependent activity modulation. Variations in GS and NR activity patterns and protein abundances were less pronounced under light-limited conditions indicating that cellular energetics and/or availability of carbon skeletons from photosynthesis may also function in the modulation of gene expression in diatoms. FCP abundance did not exhibit any significant variation with N-source but was higher in light limited cells.

**SIGNIFICANCE:** This study has provided critical information on the pathways modulating gene expression in marine diatoms by light and nitrogen supply. Our results have indicated a strong diurnal component in the expression of NR and FCP and an overall light-stimulation of mRNA abundance. These results suggest that homologous promoter elements may occur in these genes which confer light-dependent expression, the extent of expression may then be further modulated by interactions of additional sequence elements and associated transcription factors that confer a specific response to nitrogen source availability (NR) or irradiance level (FCP). This study has also provides primary information on mechanisms integrating compartmentalized metabolic functions such as carbon and nitrogen assimilation. As an initial attempt to delineate signal transduction pathways in marine diatoms from the effector end, this research will provide essential information necessary for our understanding of environmental regulation of diatom growth in biofilms as well as for our exploitation of these organisms in biotechnological applications.

**FUTURE WORK:** The immediate goal is to finish characterization of the NR, GS and FCP genes in S. costatum to provide information on their promoter structure for use in ongoing experiments entailing transformation of marine diatoms. NR in a warm-water species, Skeletonema tropicum, will be biochemically and molecularly characterized in order to identify structural motifs which may contribute to the thermal stability of this enzyme. Coastal and oceanic species will also be assayed for NR sensitivity to metal inhibition.

## PUBLICATIONS AND PRESENTATIONS

1. Gao, Y., G.J. Smith and R.S. Alberte. 1993. Nitrate reductase from the marine diatom Skeletonema costatum: biochemical and immunological characterization. Plant Physiology accepted.
2. Gao, Y., G.J. Smith and R.S. Alberte. 1992. Isolation and characterization of nitrate reductase from the marine diatom Skeletonema costatum. Presented at the 6<sup>th</sup> Congress of the Federation of Asian and Oceanian Biochemists. October 1992, Shanghai, China.
3. Robertson, D.L., G.J. Smith and R.S. Alberte. 1992. Glutamine synthetase expression is influenced by environmental nitrogen source. Presented at the 2nd Annual Monterey Bay Research Symposium. November 1992, Pacific Grove, CA.
4. Smith, G.J., Y. Gao and R.S. Alberte. 1992. Molecular probes of nitrate assimilation in marine diatoms. Presented at the 2nd Annual Monterey Bay Research Symposium. November 1992, Pacific Grove, CA.